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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,266,174,** on March 18, 1999, by **HEMOSOL INC.**, assignee of Gordon W. Adamson and Greg Angus McIntosh, for "Hemoglobin-Antioxidant Conjugates".

PRIORITY DOCUMENT

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April 3, 2000

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(CIPO 68)

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HEMOGLOBIN-ANTIOXIDANT CONJUGATES

FIELD OF THE INVENTION

This invention relates to hemoglobin compositions, and more specifically to hemoglobin-antioxidant compositions for administration to living beings for oxygen-transport purposes, antioxidant therapeutic purposes, etc.

BACKGROUND OF THE INVENTION

Hemoglobin in blood is contained within the red blood cells of the blood, in which it circulates through the body to fulfil its oxygen-transporting function. Hemoglobin in the red cells binds oxygen as the blood circulates through the lungs, delivers the oxygen to the body tissues and releases it there, for normal metabolic functions. The chemical behavior of hemoglobin in blood is constrained by its presence in the red cells, which also contain many other components such as enzymes which influence the chemical behavior of hemoglobin therein. When hemoglobin is extracted from red cells and purified ready for use as an acellular oxygen-transporter in blood substitute applications (hemoglobin-based oxygen carriers, HBOCs), the chemical influence on the hemoglobin of the other red cell components is lost.

One of these influences relates to oxygen-hemoglobin reactions, and the generation of toxic oxygen species. Oxidation of hemoglobin by liganded oxygen produces met-hemoglobin, in which heme iron is oxidized to the Fe (III) state, and in which the oxygen free radical "superoxide", $\cdot O_2^-$ is generated. Whilst met-hemoglobin is biologically tolerable, it does not have any significant useful function, since it is incapable of binding and

transporting oxygen. Superoxide is, however, linked to a number of deleterious effects in the body, such as oxidative damage and injury to vascular components including endothelium. In the red blood cell, enzymes are present to convert these undesirable oxidation products to harmless by-products. Thus, the met-reductase enzymatic system is present to reduce the met-hemoglobin to hemoglobin. Superoxide dismutase and catalase are present, respectively to convert superoxide to hydrogen peroxide, and to convert hydrogen peroxide to water and molecular oxygen.

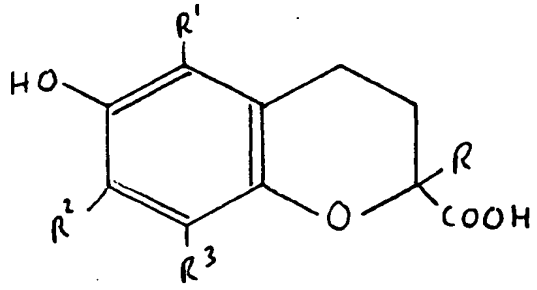
Hemoglobin outside the red cell has no such enzymatic reagents at hand to deal with these oxidation by-products. Consequently, the use of acellular hemoglobin as an oxygen-transporter may produce excessive quantities of deleterious oxidation products such as superoxide. This problem is particularly acute in situations of ischemia-reperfusion, encountered, for example, during medical operations involving the temporary interruption of blood flow to a body organ while it is surgically treated or repaired. It is known that large quantities of superoxide are generated on reperfusion of ischemic tissues with oxygen-containing solutions. Use of an acellular HBOC can accordingly be problematic in such circumstances.

It is an object of the present invention to provide a novel hemoglobin composition which overcomes or at least diminishes the above problem.

SUMMARY OF THE INVENTION

The present invention provides a hemoglobin-antioxidant composition, in the form of a chemical conjugate of a hemoglobin species and a chroman carboxylic acid having

antioxidant properties and corresponding to the general formula:



where R is an alkyl radical of 1-20 carbon atoms or an alkenyl radical of 2-20 carbon atoms, R_1, R_2 and R_3 are independently selected from H and C_1-C_4 alkyl, and R_4 is a direct bond or C_{1-8} alkyl.

The chroman-carboxylic acid is chemically covalently bound to the hemoglobin using its carboxyl function. The bonding may be direct, to primary amine groups on the globin chains of hemoglobin. Alternatively an appropriate chemical linker may be used. The chroman-carboxylic acids used in the present invention are in many cases known as bioacceptable antioxidants, capable of scavenging superoxide and other reactive oxygen species formed in vivo. It has been found according to the present invention that the antioxidant function of the chroman-carboxylic acids remains substantially unimpaired following conjugation to the hemoglobin species. The conjugates of the present invention retain oxygen-transporting capability. This is especially important since modified hemoglobins are known to extravasate, and so the antioxidant activity will be transported to any sites to which the HBOC moves.

Conjugates of the present invention provide the

antioxidative functionality in chemically bound proximity to the hemoglobin. Accordingly, the reactive oxygen species generated by oxygen-hemoglobin reaction are immediately subject to the effects of the antioxidant function, a highly desirable feature since the oxygen species are short lived and do not travel far before causing damage.

BRIEF REFERENCE TO THE DRAWING

Figure 1 is a graphical presentation of the results of Example 2 below;

Figures 2 and 3 are graphical presentations of the results of Example 4 below;

Figures 4 and 5 are similar graphical presentations of the results of Example 5 below;

Figures 6 and 7 are similar graphical presentations of the results of Example 7 below.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The cell-free hemoglobin species for use in conjugates of the present invention may be substantially any biocompatible hemoglobin capable of oxygen transport in the living mammalian system. It may be of human or animal origin. Thus it may be obtained from mammalian red blood cells, e.g. outdated human blood, by lysis of the red cells and separation and purification of the hemoglobin so obtained, by methods known in the art. The resultant hemoglobin should be stroma free and endotoxin free, for best biocompatibility. Alternatively, the hemoglobin may be prepared, in native or mutant form, by recombinant techniques and cell culture techniques known in the art. The use of natural or unnatural mutant hemoglobin species is also within the scope of the invention.

A preferred form of hemoglobin for use in the present invention is cross-linked hemoglobin, in which the tetrameric hemoglobin units have been chemically intramolecularly cross-linked to prevent dissociation into hemoglobin dimers. As is well known, this tendency for dissociation of natural hemoglobin tetramers into dimers is another consequence of extracting hemoglobin from the red cells of blood. Hemoglobin dimers formed by such dissociation, of molecular weight about 32,000 Daltons, are prematurely lost from the system by excretion through the kidney, and so dissociation should be minimized. A variety of methods are known and disclosed in the art for intramolecularly cross-linking hemoglobin to guard against such dissociation, using a variety of chemical cross-linkers such as glutaraldehyde, polyaldehydes such as those derived from ring opening oxidation of sugars and polysaccharides diaspirin compounds, pyridoxyl compounds, trimesoyl compounds, and the like. The hemoglobin used in the present invention may also be polymerized by intermolecular linking of two or more such tetramers, preferably up to eight such tetramers, into a polymeric form, using the same or multiple cross-linking reagents. Mixtures containing two or more different such species of intramolecularly cross-linked and intermolecularly linked hemoglobin are particularly desirable. The chemical reactions to effect cross-linking and, optionally, polymerization are preferably conducted before conjugation of the chroman-carboxylic acid with the hemoglobin species.

The present invention can also be used with other modified forms of hemoglobin, such as hemoglobin conjugated to polymers, e.g. appropriately functionalized polyethylene oxide (PEG), polysaccharides, polyamino acids and insoluble supports. All can benefit from the presence of antioxidant

molecules bonded thereto, as described herein.

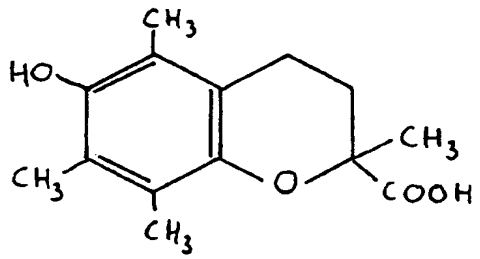
In an alternative according to the present invention, the chroman-carboxylic acid is coupled to a non-cross-linked hemoglobin, and cross-linking of the conjugate is subsequently undertaken, to form intramolecularly stabilized tetrameric hemoglobin-antioxidant complexes, optionally in admixture with oligomerized or polymerized such complexes, containing up to about 8 chemically bonded tetrameric hemoglobin-antioxidant species. The cross-linking reagent used in such a procedure can be any of those mentioned above, although oxidatively ring-opened raffinose is preferred, on account of the desirable product composition which it yields. The conditions of the hemoglobin cross-linking reaction, when conducted after conjugation to the chroman-carboxylic acid antioxidant, are not significantly different from those utilized for cross-linking hemoglobin alone.

Using either strategy, whereby hemoglobin is conjugated to the chroman-carboxylic acid prior to cross-linking of the hemoglobin, or cross-linked hemoglobin is conjugated to the chroman-carboxylic acid, any non-crosslinked hemoglobin will be modified with the chroman-carboxylic acid. This is beneficial since the non-cross-linked hemoglobin is still capable of generating reacting reactive oxygen species, and this form of hemoglobin is known to have different biodistribution properties in comparison with cross-linked hemoglobins.

The chroman-carboxylic acid used in conjugates of the present invention and corresponding to the above chemical formula may be a vitamin E carboxylic acid derivative, e.g. one in which radical R is a branched alkyl or alkylene chain of 16 carbon atoms, such as 4,8,12-trimethyl-tridecyl or

4,8,12-trimethyl-3,7-11-tridecatrienyl, with any of the various possible stereoconfigurations. Compounds in which at least one of R_1, R_2 and R_3 is methyl, and R_4 is a direct bond are preferred. Another preferred group of compounds is those of the above formula in which R represents methyl.

Most preferred among chroman-carboxylic acids for use in the present invention is 2,5,7,8-tetramethyl-2-carboxy-chroman-6-ol, commonly known as Trolox, of chemical formula:



The invention will accordingly be further described with specific reference to the use of Trolox, for ease of description, but this should not be construed as a limitation.

The hemoglobin and Trolox can be chemically bonded together. The carboxyl function of the trolox residue reacts with a primary amine group on a globin chain of hemoglobin, e.g. a lysine residue, to form a covalent amide bond.

The reaction of trolox and the hemoglobin may be facilitated by the use of an activating chemical compound such as 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC) or other carbodiimides (alone or in combination with other activators such as N-hydroxysulfosuccimide), isoxazolium derivatives such as Woodward's reagent K, chloroformates, N,N'-carbonyldiimidazole, N-carbalkoxydihydroquinolines and the

like. The trolox may be used in acid, acid derivative or anhydride form. Activating compounds such as EDC react first with the trolox to activate the trolox carboxyl group, which then reacts with an amino group of hemoglobin, with elimination of the EDC functionality. The use of such activating compounds allows for larger loadings of trolox onto hemoglobin, and better control over the amount of such loading.

The conditions and procedures for reacting hemoglobin which such carbodiimide compounds are well within the skill of the art. Reactions suitably take place at room temperatures, using aqueous solutions.

Instead of direct bonding, a chemical spacer or linker may be utilized, so that the conjugate comprises hemoglobin to which one or more non-trolox molecules are bonded, and trolox is bonded to the non-trolox chemical residues. Examples of such linkers include functionalized sugars and polysaccharides, polyamino acids such as polylysine, PEG derivatives, and various bifunctional linkers. The use of such linkers can provide several trolox attachment sites per bond to hemoglobin, to provide greater loading with trolox with less modification of the hemoglobin. It also allows various modifications to the properties of the conjugates (solubility, activity, etc.) by choice of appropriate linker.

The precise group or groups on the globin chains which are used to bind to the trolox, optionally through the linker, do not appear to be critical. The sites may be on either or both of the alpha globin chains and the beta globin chains. Accordingly, selectivity of the conjugation reaction is not an important factor.

A preferred feature of the process of the invention is the addition of the desired quantity of trolox in several sequential aliquots, e.g. 2 - 5, instead of as a single addition of the entire amount. Such sequential addition leads to a larger loading of trolox onto hemoglobin, and to resultant products with greater antioxidant activity.

The preferred amount of trolox conjugated to hemoglobin according to the present invention is determined on the basis of providing sufficient trolox to perform its antioxidant, radical scavenging function in practice, but not so much as to interfere with the oxygen transporting capability and oxygen affinity of the hemoglobin. The amount can be controlled by control of the amount of activating material and/or trolox added to the reaction solution in which the conjugate is made. Suitable such relative amounts of hemoglobin and trolox are from about 1 to about 100, with the most preferred amounts being from about 10 to about 100.

After preparation of the conjugate as described, the product is carefully and thoroughly purified to remove unchanged reagents and any other contaminants. Purification may be by chromatography (size exclusion, HIC, affinity, ion exchange, etc.) or other methods known in the art, including dialysis/diafiltration, ultrafiltration, or selective precipitation, centrifugation, extraction or any other form of separation. The conjugate is suitably stored under sealed, non-oxidative conditions, as an aqueous solution ready for administration to a patient as required.

The invention is further described, for illustrative purposes, in the following non-limiting specific examples:

SPECIFIC DESCRIPTION OF THE MOST PREFERRED EMBODIMENTS

EXAMPLE 1 - Preparation and characterization of Conjugates

A series of experiments was conducted in which Trolox (TX) was conjugated to carbonmonoxyhemoglobin (COHb) using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) as a coupling agent under different conditions set out in Table 1 below. In each case, EDC (1.05 g, 5.46 mmole) was added to a suspension of Trolox (TX, 1.36 g, 5.45 mmole) in 3.50 mL acetonitrile (AcN), giving 1.55 M TX-EDC reagent. After 10 minutes, 10- and 100-fold dilutions were made in acetonitrile, providing 155 mM and 15.5 mM reagent, respectively. Reaction mixtures were prepared according to table 1 and held at 22°C for up to 24 hours under CO gas. Unless otherwise noted, stock Hb was 3.27 mM and buffer was 100 mM MES (pH 7.0). Any precipitate was removed by centrifugation or filtration prior to analysis. Samples were diluted to approximately 10 microM Hb for reverse phase HPLC analysis. Conjugates were dialyzed against phosphate-Buffered saline (PBS), pH 7.4, prior to antioxidant activity assay.

TABLE 1: PREPARATION OF TROLOX-Hb REACTION SOLUTION

Rxn	TX:Hb ratio	Stock TX-EDC (M)	Stock COHb (mL)	Stock TX-EDC (mL)	H2O (mL)	Buffer (mL)	Final TX-EDC (M)	Final Hb (mM)
1	100	1.55	1.90	0.40	0	1.70	0.155	1.55
2	100	1.55	1.90	0.40	0	1.70*	0.155	1.55
3	100	1.55	1.90	0.40	0	1.70	0.155	1.55
4	10	0.155	1.90	0.40	0	1.70	0.015	1.55
5	1	0.0155	1.90	0.40	0	1.70	0.00155	1.55
6	0	0	1.90	0.40	0	1.70	0	1.55
7	100	0.155	0.95	2.00	8.55	8.50	0.0155	0.155
8	10	0.0155	0.95	2.00	8.55	8.50	0.00155	0.155
9	0	0	0.95	2.00	8.55	8.50	0	0.155

*Buffer was 100 mM MES (pH 6.0), all other reactions at pH 7.0

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For characterization of the conjugates so prepared, reverse phase HPLC was used to separate the globin chains (native or modified) of conjugates. Heme is also separated during this process. Integrated areas of ultraviolet light absorption peaks of the eluting globin chains were used to calculate the relative proportions of chains, and electrospray mass spectrometry coupled to reverse phase HPLC (LCMS) was used to determine molecular weights of the chains (Table 2). Typically, modified chains eluted later than unmodified chains. Three major modified chains were identified by LCMS (Table 3): beta chain with one trolox molecule attached ($\beta(TX)_1$), and alpha chains with one ($\alpha(TX)_1$) or two ($\alpha(TX)_2$) trolox molecules attached. Masses are in agreement with amide-linked conjugates.

TABLE 2: Degree of Hb modification by TX-EDC under various conditions

Rxn	Conjuated TX:Hb ratio*	Hb concn (mM)	TX:Hb ratio	Rxn pH	Rxn time (hr)	Modified chains (%)	$\alpha(TX)_1$	$\alpha(TX)_2$	$\beta(TX)_1$	Other (%)
1	1.6	1.55	100	7	4	43.7	26.4	3.9	5.4	7.9
2	2.0	1.55	100	6	4	52.6	33.6	3.6	8.2	7.2
3	1.9	1.55	100	7	24	50.5	32.8	4.7	4.8	8.2
4	0.7	1.55	10	7	24	24.0	12.4	0.0	6.0	5.6
5	0.1	1.55	1	7	24	1.5	1.5	0.0	0.0	0.0
6	0.0	1.55	0	7	24	0.0	0.0	0.0	0.0	0.0
7	3.7	0.155	100	7	24	79.5	26.3	21.0	24.5	7.6
8	0.8	0.155	10	7	24	22.0	13.9	1.1	4.0	3.1
9	0.0	0.155	0	7	24	0.00	0.0	0.0	0.0	0.0

*Conjugated TX:Hb ratio does not include uncharacterized species listed as "other"

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Table 3: Calculated and observed masses for globin chains of Hb-TX conjugates

Globin chain	Calculated mass (Da)	Observed mass (Da)
α	15126	15125
β	15868	15864
$\alpha(TX)_1$	15358	15358
$\alpha(TX)_2$	15591	15595
$\beta(TX)_1$	16100	16099

EXAMPLE 2 - Measurement of Antioxidant Activity

Blood was collected into heparinized tubes and erythrocytes were separated by centrifugation and washed 3 times with 10 volumes of saline. During the last washing, erythrocytes were centrifuged at 1000x g for 10 minutes to obtain a consistently packed cell preparation. The assay for hemolysis mediated by peroxyl radicals was conducted by a modified method of Miki et al.,(M. Miki, H. Tamai, M. Mino, Y. Yamamoto and E. Niki., Arch. Biochem. Biophys. 258:373-380 (1987)). Equal volumes of a 30% suspension of fresh erythrocytes in PBS pH 7.4, test sample, and 300 mM 2,2;-azo-bis(2-amidinopropane dihydrochloride)(AAPH, a radical generator) were combined in order. Mixtures were held at 37°C, and aliquots were diluted 20-fold in PBS and centrifuged at 1000x g for 10 minutes. Absorbances (414 nm) of supernatants were determined as measures of Hb released due to RBC lysis. Supernatant Hb levels were corrected for the presence of the added test sample Hb. The results are presented graphically in Figure 1. Products analyzed were prepared as described for reaction #2 (Hb-TX 1) and reaction #3 (Hb-TX 2) in Table 1 of Example 1. In the presence of Hb controls (no conjugation of Trolox), RBC lysis is evidenced

by increasing levels of Hb in supernatant over the incubation period. Supernatant Hb levels do not increase to the same level over this period in test mixtures containing Hb-TX conjugate, indicating protection against the lytic effect of the radical generator.

Example 3 - Trolox-polymerized Hb conjugate preparation

Trolox (TX) was conjugated to o-raffinose cross-linked Hb (polyOR-Hb, U.S.Patent 5,532,352 Pliura et al.). A 100-fold molar excess of TX-EDC was reacted with polyOR-Hb in 100 mM MES buffer at pH 5,6 and 7. Control reactions of polyOR-Hb reacted with either TX or EDC (the coupling agent) alone were run. All samples were analyzed by size exclusion chromatography (SEC) and reversed phase HPLC (RP HPLC). In polyOR-Hb, alpha and beta chains are 33 and 90% modified by o-raffinose, respectively (Table 4). After reaction with TX at three different pH values, alpha and beta chain modification was increased to 67-77% and 93-99%, respectively, and several new modified chains were observed by RP HPLC. Several of these TX-modified chains corresponded to those observed after reaction of Hb with TX-EDC, as identified in Example 1.

Table 4 - Modification of globin chains of polyOR-Hb-TX conjugates

Rxn	Sample	Rxn pH	% Modn of Globin compared to HbAo		Increase in % Modn of Globin Compared to polyOR-Hb	
			Beta	Alpha	Beta	Alpha
1	HbAo	n/a	n/a	n/a	n/a	n/a
2	polyOR-Hb	n/a	90.2	33.3	n/a	n/a
3	polyOR-Hb-TX	7	93.4	75.6	3.2	42.3
4	polyOR-Hb-TX	6	99.7	77.1	9.5	43.8
5	polyOR-Hb-TX	5	96.6	67.3	6.4	34.0

Example 4 : (i) Conjugation of Trolox to hemoglobin at various concentrations

To 1.0 g CO-Hb in 99 mL 54 mM MES buffer pH 7.0 was added 0.39 g Trolox with 0.30 g EDC in 1 mL acetonitrile, for a final Hb concentration of 10 mg/mL. 1.0 g CO-Hb in 9 mL 54 mM MES buffer was similarly treated for a final Hb concentration of 100 mg/mL. Control reactions contained Hb treated with acetonitrile alone. After 20 hr mixing at 22°C, particulates were removed by centrifugation and filtration, and filtrates dialyzed against water, Tris-buffered 0.5 M $MgCl_2$, and finally phosphate-buffered saline pH 7.4. Both the 10 mg/mL solutions (Hb-Trolox A) and the 100 mg/ml solutions (Hb-Trolox b) were adjusted to 43 mg/mL prior to analysis. Reverse phase HPLC analysis indicated extensive modification of alpha and beta globin chains in Trolox-treated samples, with no evidence of modification in samples treated only with acetonitrile. The results are shown in the following Table 5.

Table 5: Globin chain modification by Trolox during reactions at various Hb concentrations

Product	Modified chains	$\alpha(TX)_1$ (%)	$\alpha(TX)_2$ (%)	$\beta(TX)_1$ (%)	Other (%)	Conjugated TX:Hb ratio*
Hb-Trolox A	59.4	25.3	3.9	10.4	19.8	1.7
Hb-Trolox B	66.7	35.6	7.0	11.5	12.6	2.4

*Conjugated TX:Hb ratio does not include uncharacterized species listed as "other"

ii) In vitro protection of RBCs against lysis:

Using the method described in Example 2, but measuring absorbance at 540 nm (which also indicates levels of Hb in supernatant resulting from cell lysis), the anti-oxidant activities of the Hb-Trolox were measured. Hb was CO-ligated and concentration in the RBC assay suspension was 12.7 mg/mL. Both Hb-Trolox conjugates showed greater protection than hemoglobin controls without Trolox. Trolox conjugation resulted in an approximate 2-fold increase in the time of onset of lysis versus controls, as shown in Figure 2. Protection was greatest in the product of the 100 mg/mL reaction (Product B), which was more extensively modified by Trolox than the product of the 10 mg/mL reaction (Product A). Relative protection was also determined by comparison of areas under the curves (AUC) obtained by plotting RBC lysate absorbances versus time. Lower AUC values indicate greater protection of RBCs against lysis. AUCs for Products A and B were 25% and 11% of the AUCs for their respective controls, as shown on Figure 3.

Example 5:

i) Conjugation of Trolox to o-raffinose polymerized hemoglobin: multiple additions of Trolox

o-Raffinose polymerized hemoglobin (polyOR-Hb) was prepared as described in US Patent 5,532,352 Pliura et al.

To each of two separate solutions (Reactions A and B) of 0.50 g polyOR-Hb in approximately 50 mL 125 mM MES buffer, pH 7.0 was added 0.194 g Trolox with 0.149 g EDC in 1 mL acetonitrile. Identical amounts of Trolox/EDC were added to Reaction B at 5 and 19 hours, for a total of three additions. Reaction A had 1 mL volumes of acetonitrile added at the same times. Both reactions were stirred under CO gas at 22°C for a total of 27 hours. Particulates were removed by centrifugation, and low molecular weight solutes (unreacted Trolox, EDC and MES) removed by dialysis against water, Tris-buffered 0.5 M $MgCl_2$ and phosphate buffered saline pH 7.4. No free Trolox was detectable by chromatography. Control products were prepared in the same manner, except that no Trolox was added.

ii) <u>In vitro protection of RBCs against lysis:</u>

Using the method described in Example 2, the anti-oxidant activities of the two conjugates were measured. Samples were CO-ligated and concentration of the conjugates and controls in the RBC assay suspension was 12 mg/mL. Both Trolox conjugates showed greater protection than corresponding controls without Trolox . This is shown on Figure 4, a set of curves derived as described in the previous example. Protection was greatest in the product obtained after three additions of Trolox, which was shown by reverse phase HPLC analysis to be more extensively modified by Trolox than the product obtained by a single addition of Trolox. AUC for the 3-fold addition product was 3% of control, while AUC of the single addition product was 33% of control - see accompanying Figure 5.

Example 6: Polymerization of hemoglobin modified with Trolox

Hemoglobin-Trolox conjugates prepared in Example 4 were dialyzed against 50 mM Bis-Tris buffer, pH 6.8. Three equivalents o-raffinose dissolved in water were added to solutions of hemoglobin-Trolox to give a final hemoglobin concentrations of 42 mg/mL. The mixtures were held under CO gas at 22°C for 24 hours. The solutions were made 30 mM in sodium acetate, and 20 equivalents of aqueous dimethylamine borane relative to o-raffinose content were added. After 24 hours, the solutions were dialyzed against water then phosphate-buffered saline pH 7.4. Size exclusion chromatography indicated formation of intra- and intermolecularly crosslinked hemoglobin-Trolox species (Table 6).

Table 6: Molecular weight distribution of o-raffinose polymerized hemoglobin-Trolox

Molecular weight species (kDa)	Molecular weight distribution (%)	
	Polymerized Hb-Trolox A	Polymerized Hb-Trolox B
32	12.2	11.8
64	44.7	41.1
>64	43.1	47.1

Example 7:

i) Large scale preparation of Trolox conjugate of o-raffinose polymerized hemoglobin (PolyOR-Hb-TX):

o-Raffinose polymerized hemoglobin (polyOR-Hb) was prepared as described in US Patent 5,532,352 Pliura et al. To 18.9 g polyOR-Hb in 2 L 126 mM MES buffer, pH 7.0 was added a solution of 4.01 g Trolox with 3.07 g EDC in 40 mL acetonitrile. Identical additions of Trolox/EDC were made after 3.5 and 21 hours, for a total of three additions. The reaction was stirred under CO gas at 22°C throughout the process. After 26 hours total reaction time, particulates were removed by filtration, and low molecular weight solutes (unreacted Trolox, EDC and MES) removed by diafiltration against water, phosphate-buffered saline, and Ringer's lactate. The pH was adjusted to 7.24 with dilute NaOH during the Ringer's lactate diafiltration. No free Trolox was detectable by chromatography. A portion of the product was oxygenated prior to further analysis.

ii) In vitro protection of RBCs against lysis:

Using the method described in Example 2 but using absorbance at 540 nm as previously described, the anti-oxidant activity of the polyOR-Hb-TX was measured. Products tested included oxygen and CO-ligated polyOR-Hb (no Trolox attached), CO-ligated product reserved prior to oxygenation as described above, and oxygenated product. All products were present in the RBC lysis assay suspension at 11.7 mg/mL. The results are presented graphically on Figure 6 and Figure 7. These results indicate better protection by both the oxygenated and carbonmonoxy forms of Trolox conjugates than by controls.

Example 8

Hemodynamic effect of polyOR-Hb-TX following 10% topload infusion in conscious rat

Male Sprague-Dawley rats (250-350 g) were anesthetized with isoflurane on the day of the experiment. The right femoral artery and vein were cannulated. After 1.5 hour recovery from surgery, conscious animals residing in a metabolic cages were infused with either of two solutions: polyOR-Hb-TX prepared in Example 7 or polyOR-Hb (both solutions were 7.7 g/dL in lactated Ringer's solution). Infusion volume was equal to 10% of the animal's estimated blood volume. Mean arterial blood pressure (MAP) and heart rate (HR) were recorded 30 minutes prior to infusion to establish baseline values, during infusion and for 2 hours following infusion (Figures 8 and 9). Four animals were tested in each of the two groups. The baseline MAP prior to infusion was 101±4 mm Hg (polyOR-Hb) and 110±3 mm Hg (polyOR-Hb-TX). Following infusion, MAP increased significantly

(P<0.01) to 142±7 and 151±1 mm Hg in the polyOR-Hb and polyOR-Hb-TX groups, respectively. The difference in increase was not significantly different between the two groups (P>0.05). Pre-infusion HR were 407±17 and 394±17 beats per minute (bpm) in the polyOR-Hb and polyOR-Hb-TX groups, respectively. HR after infusion decreased significantly (P<0.01) to 345±16 and 316±10 bpm in the polyOR-Hb and polyOR-Hb-TX groups, respectively. The difference in decrease was not significantly different between the two groups (P>0.05). Conjugation of Trolox did not alter the hemodynamic properties of the HBOC polyOR-Hb in this study.

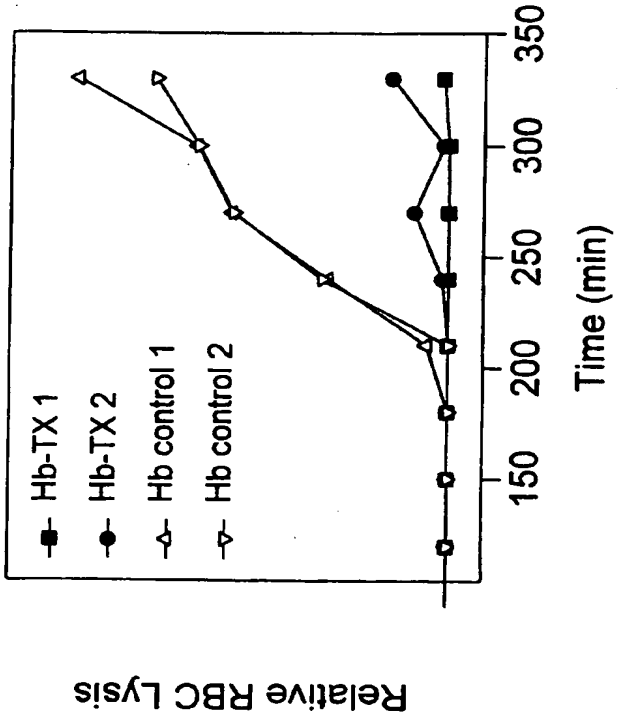

Figure 1: Radical-mediated red blood cell lysis in the presence of conjugated and non-conjugated Hb.

FIG. 2

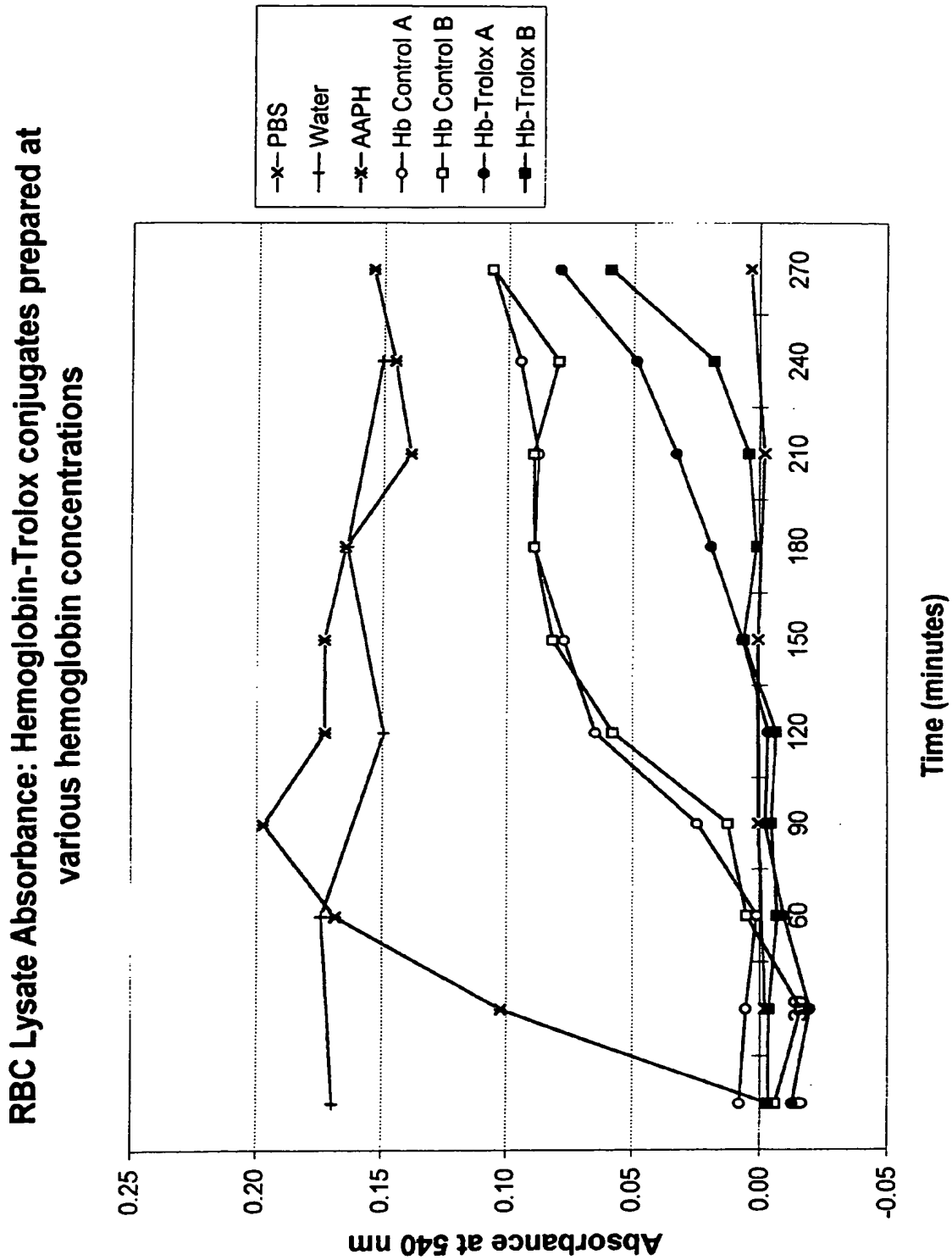
RBC Lysate Absorbance: Hemoglobin-Trolox conjugates prepared at various hemoglobin concentrations
PBS
Water
AAPH
Hb Control A
Hb Control B
Hb-Trolox A
Hb-Trolox B
Absorbance at 540 nm
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
Time (minutes)
0
30
60
90
120
150
180
210
240
270

FIG. 3

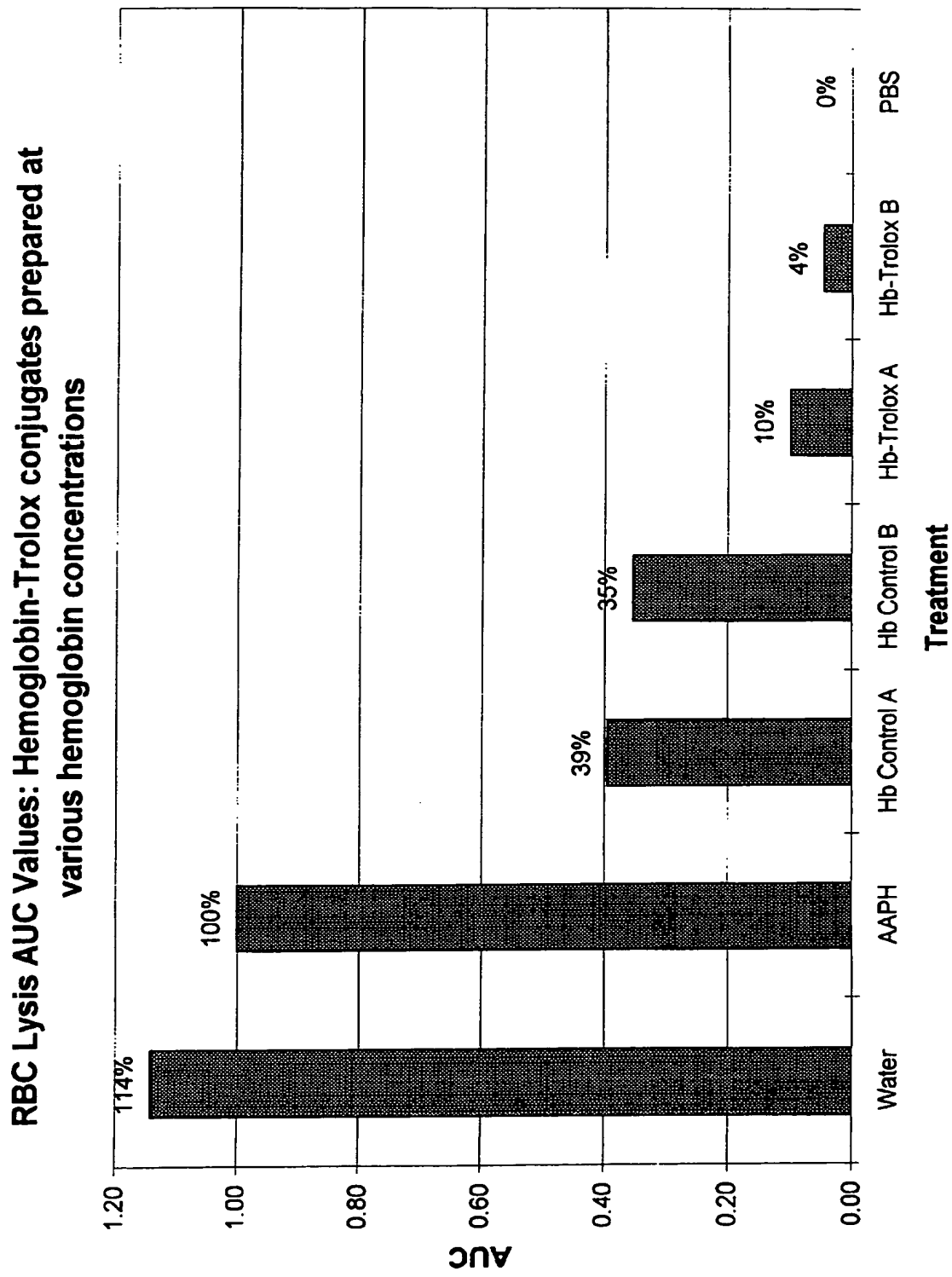
RBC Lysis AUC Values: Hemoglobin-Trolox conjugates prepared at various hemoglobin concentrations
AUC
1.20
1.00
0.80
0.60
0.40
0.20
0.00
114%
100%
39%
35%
10%
4%
0%
Water
AAPH
Hb Control A
Hb Control B
Hb-Trolox A
Hb-Trolox B
PBS
Treatment

FIG. 4

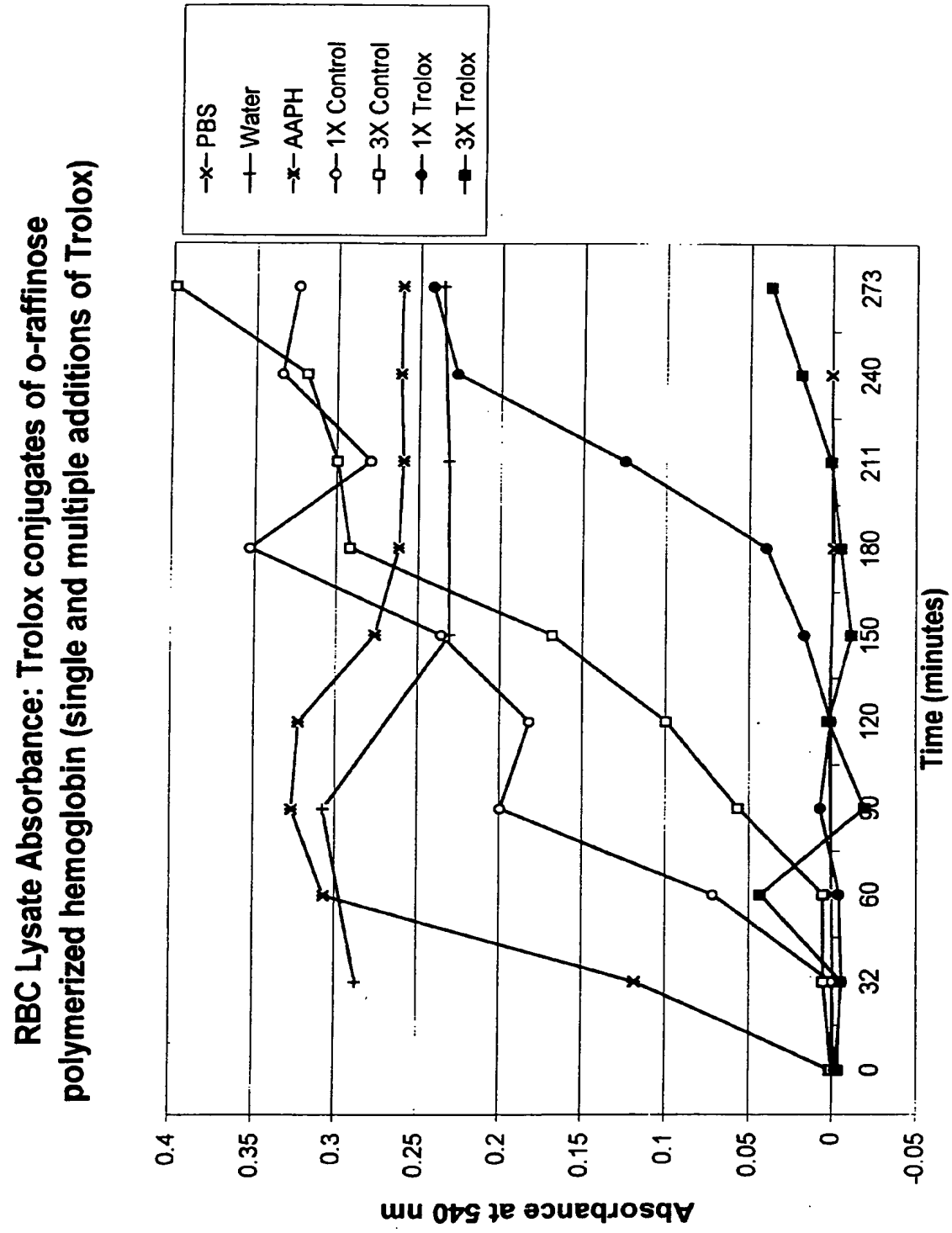
RBC Lysate Absorbance: Trolox conjugates of o-raffinose polymerized hemoglobin (single and multiple additions of Trolox)
PBS
Water
AAPH
1X Control
3X Control
1X Trolox
3X Trolox
Absorbance at 540 nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
32
60
90
120
150
180
211
240
273
Time (minutes)

FIG. 5

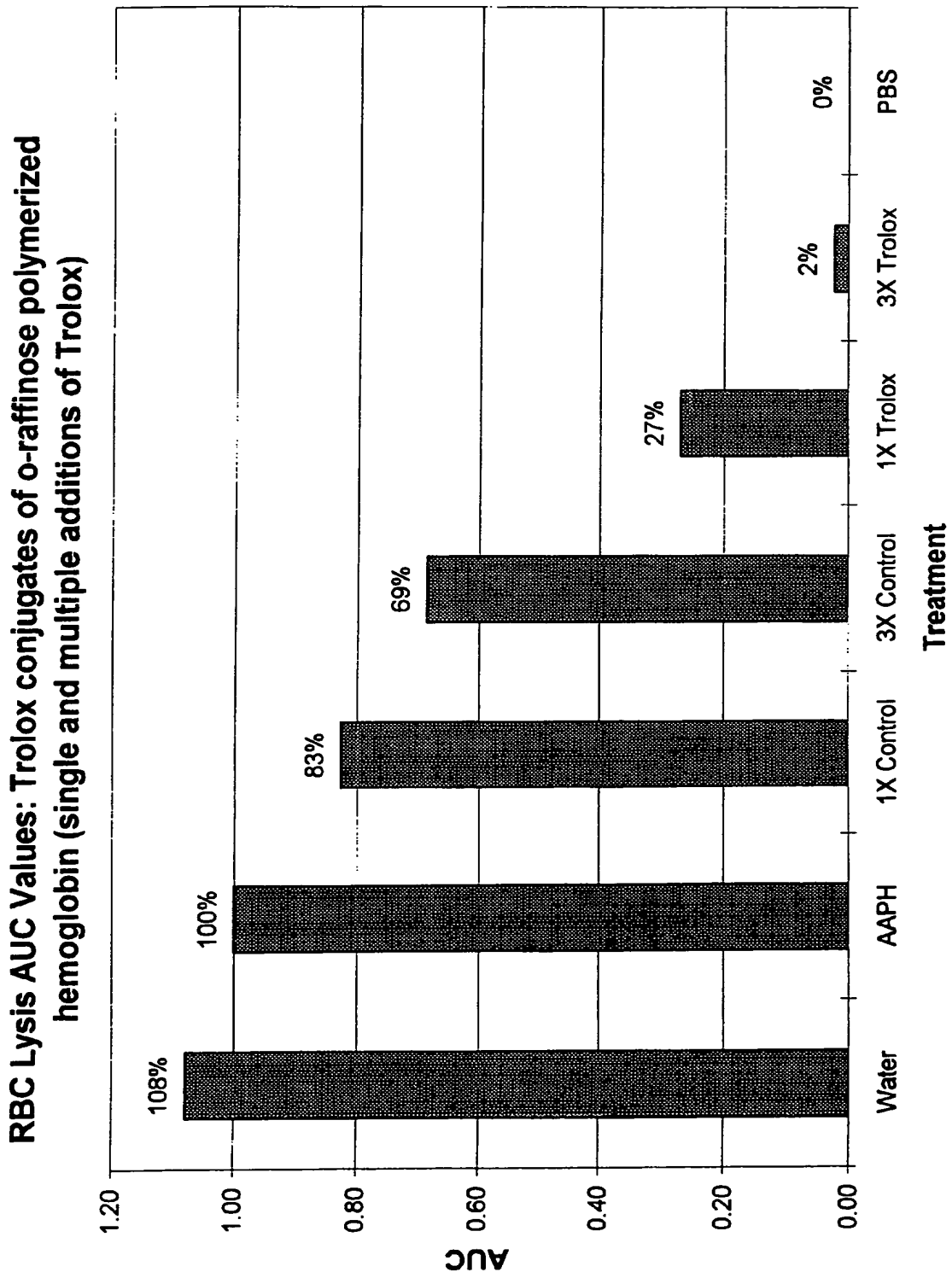
RBC Lysis AUC Values: Trolox conjugates of o-raffinose polymerized hemoglobin (single and multiple additions of Trolox)
AUC
1.20
1.00
0.80
0.60
0.40
0.20
0.00
108%
100%
83%
69%
27%
2%
0%
Water
AAPH
1X Control
3X Control
1X Trolox
3X Trolox
PBS
Treatment

FIG. 6

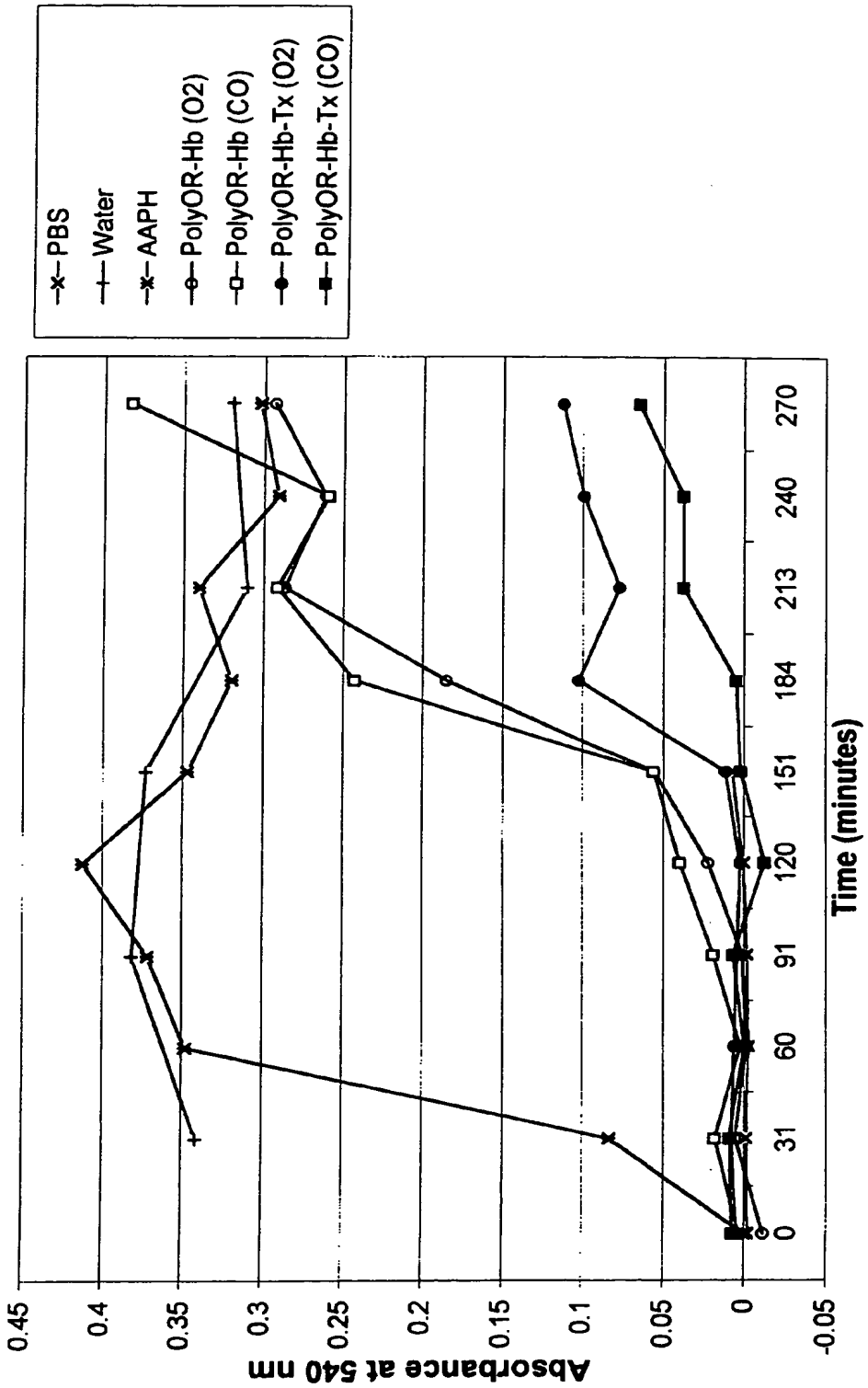
RBC Lysate Absorbance: Trolox conjugate of o-raffinose polymerized hemoglobin (large scale preparation)
PBS
Water
AAPH
PolyOR-Hb (O2)
PolyOR-Hb (CO)
PolyOR-Hb-Tx (O2)
PolyOR-Hb-Tx (CO)
Absorbance at 540 nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
31
60
91
120
151
184
213
240
270
Time (minutes)

FIG. 7

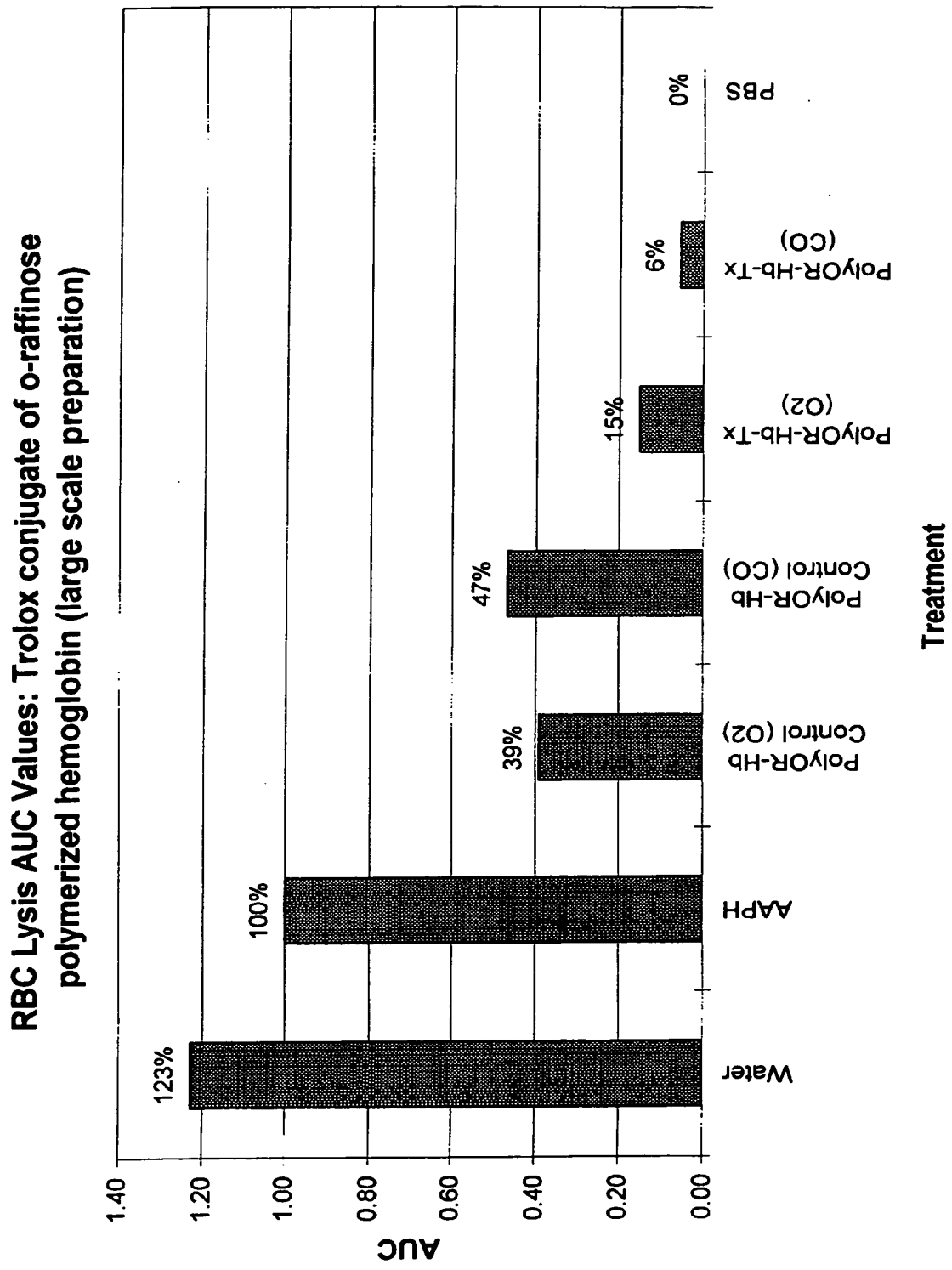
RBC Lysis AUC Values: Trolox conjugate of o-raffinose polymerized hemoglobin (large scale preparation)
AUC
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
123%
100%
39%
47%
15%
6%
0%
Water
AAPH
PolyOR-Hb Control (O2)
PolyOR-Hb Control (CO)
PolyOR-Hb-Tx (O2)
PolyOR-Hb-Tx (CO)
PBS
Treatment

Figure 8: 10% Topload infusion in conscious rat: Mean arterial pressure (Mean±SEM, n=4)

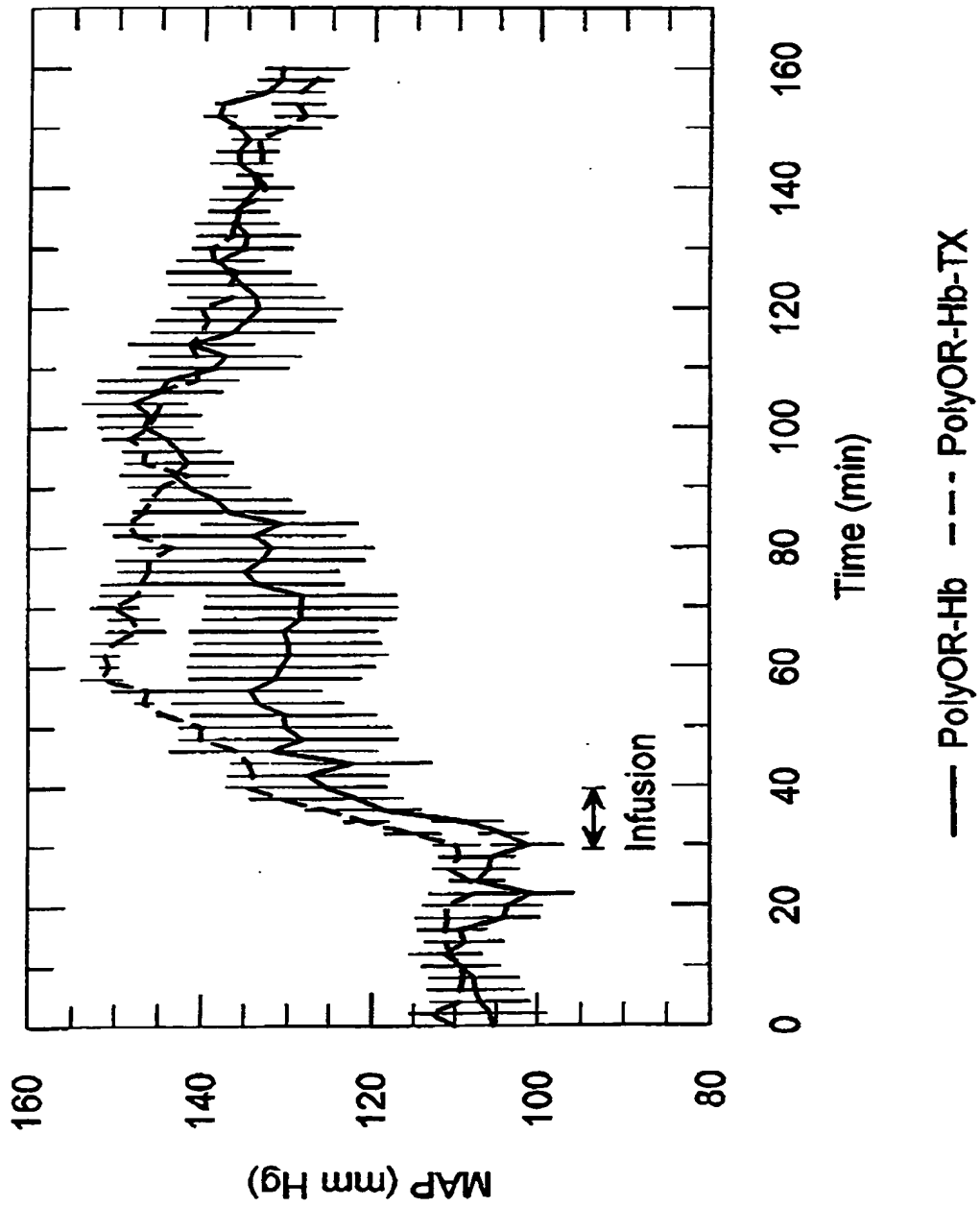

Figure 9: 10% Topload infusion in conscious rat: Heart rate (Mean±SEM, n=4)